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Original article

Mutation analysis of tumor necrosis factor alpha-induced protein 3 gene in Hodgkin lymphoma

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ABSTRACT

Aims: Survival and proliferation of Hodgkin and Reed-Sternberg (HRS) cells, the malignant cells of classical Hodgkin lymphoma (CHL), are dependent on constitutive activation of nuclear factor kB (NF-κB). A20, encoded by TNF alpha-induced protein 3 ($TNFAIP_3$), one of the inhibitors of NF-kB, was found to be inactivated by deletions and/or point mutations in CHL.
Methods: $TNFAIP_3$ mutations were examined in 37 patients with CHL by using PCR and direct sequencing. In addition, protein expression of A20 was evaluated by immunohistochemistry. Epstein–Barr virus (EBV) status of HL samples was determined by EBV EBER chromogenic in situ hybridization (ISH).
Results: We identified 8 mutation positive cases in a collective of 37 investigated cases (22%). Mutations were most frequent in the nodular sclerosis subtype. Our results revealed the tendency that cases harboring A20 mutations were negative for A20 staining. None of A20 mutation-positive CHL cases showed EBV infection.
Conclusions: Our study confirms the involvement of the $TNFAIP_3$ tumor suppressor gene in CHL. A20 may represent a suppressor of human lymphoma and provide a critical molecular link between chronic inflammation and cancer. None of A20 mutation-positive CHL cases showed EBV infection. This fact suggests complementing functions of $TNFAIP_3$ inactivation and EBV infection in CHL pathogenesis and may represent an interesting point of further investigations.

1. Introduction

Classical Hodgkin lymphoma (CHL) is one of the most common malignant lymphomas, with incidence rates of about 3 new cases per 100000 people per year in western world. HL is characterized by the presence of rare Hodgkin and Reed/Sternberg-cells (HRS, the tumor cells), which are embedded in an extensive inflammatory infiltrate. Compared with other lymphoma subtypes it shows infrequent bone marrow infiltration [1]. Patients with a history of infectious mononucleosis due to Epstein-Barr virus (EBV), a member of the gamma herpesvirus family, may have an increased risk of HL. In such cases, the HRS cells express EBV-encoded RNAs (EBERs). Constitutive activation of NF-κB in HRS cells plays a central role in the pathogenesis of HL. NF-κB family members including C-rel, RelB, RelA (p65), p105 and p100, form homo- and heterodimers which regulate transcription of a large number of genes [2].

NF-κB proteins are sequestered in the cytoplasm as latent complexes by inhibitory proteins (IκBs) that prevent NF-κB nuclear translocation and DNA binding. One of the inhibitors of NF-κB, the TNF alpha-induced protein 3 ($TNFAIP_3$), also known as the dual ubiquitinating and deubiquitinating enzyme A20, was found to be inactivated in lymphomas with constitutive NF-κB activity. Because loss of $TNFAIP_3$ also contributed to lymphoma cell survival [3,4], these findings established $TNFAIP_3$ as an important tumor suppressor gene. The A20 protein encoded by the $TNFAIP_3$ gene is a nuclear factor- κB (NF-κB)-induced, intracellular signal transduction component of the tumor necrosis factor receptor (TNFR) and Toll-like receptor pathways. The protein is a negative regulator of nuclear factor (NF)-κB signaling in multiple pathways. A20/$TNFAIP_3$ is located on chromosome 6q23 and contains a N-terminal ovarian tumor (OTU) domain and seven zinc finger domains in the C-terminus. The protein uses two opposing enzymatic functions to inhibit IKK (IκB-Kinase-complexes): (a)

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Table 1
Study cohort.

	in total HL	NSHL	MCHL	LDHL	LRHL	nosCHL
No of patients	37	18	13	1	1	4
male	20	10	7	1	0	2
female	17	8	6	0	1	2
EBV status	n = 37	n = 18	n = 13	n = 1	n = 1	n = 4
EBV positive	7	0	6	0	0	1
EBV negative	30	18	7	1	1	3
A20 mutation	8 (21,6%)	4 (22,2%)	1 (7,7%)	1 (100%)	0	2 (50%)
A20 wildtype	29 (78,4%)	14 (77,8%)	11 (92,3%)	0	1 (100%)	2 (50%)

NSCHL: nodular sclerosis classical Hodgkin lymphoma; MCHL: mixed cellularity Hodgkin lymphoma; LDHL: lymphocyte-depleted Hodgkin lymphoma; LRHL: lymphocyte-rich Hodgkin lymphoma; nos-CHL: not-otherwise-specified classical Hodgkin lymphoma .

deubiquitinating (DUB) activity mediated by the A20 N-terminal OTU-domain, and (b) ubiquitin protein ligase (E3) activity mediated by the C-terminal zinc finger (ZnF) region [5]. Inactivation of the A20 protein occurs by the TNFAIP$_3$ gene mutation in classical Hodgkin lymphoma (CHL) and non-Hodgkin lymphoma [6,7]. However, compared to non-Hodgkin lymphoma, the mutation status of A20 has not been well studied in Hodgkin lymphoma. Therefore in this study, we analyzed A20 (TNFAIP$_{3)}$ mutation together with its protein expression and EBV/EBER status in 37 patients with Hodgkin lymphoma.

2. Material and methods

2.1. Tumor collective

In total 37 cases of Hodgkin lymphoma were retrieved from the database of the Institute of Pathology, Jena University Hospital, for molecular analysis (Table 1). These cases were classified according to the current World Health Organization (WHO 2008) classification subdividing nodular lymphocyte-predominant HL (NLPHL) from classical HL (CHL) and its subtypes like nodular sclerosis, mixed cellularity, lymphocyte-depleted and lymphocyte-rich CHL. Formalin-fixed, paraffin-embedded (FFPE) tissues were available for molecular testing. The study was approved by the ethics committee of the Jena University Hospital (No. 4342-02/15).

2.2. EBV status (In situ hybridization)

EBV status of HL samples was determined by EBV EBER chromogenic in situ hybridization using sections of paraffin-embedded materials. The experiments were performed according to the manufacturers' instructions for an automated system (Bond Max, Leica Biosystems, Germany) as well as a manual system (Dako, Denmark). Appropriate positive and negative controls were included in all analyses [8].

2.3. Immunohistochemistry with A20 antibody

Immunohistochemical staining of A20 was performed as previous described [8,9]. Briefly, antigen retrieval was performed by treatment in a pressure cooker for 6 min. The rabbit anti-TNFAIP$_3$ (A20) monoclonal antibody with working concentration of 1:25 (Abcam, Cambridge, UK) was incubated at room temperature for 1 h. Detection took place according to the manufacturer's instructions (LSABTM 2-kits, Dako, Denmark). All slides were read by one pathologist (I. Petersen). Immunohistochemistry was scored positive if more than 5% of tumor cells showed nuclear staining of A20.

2.4. Mutation analysis of TNFAIP$_3$

Genomic DNA was extracted from FFPE tissues according to manufacturer's instructions (QIAGEN, Germany). Only in one case, manual microdissection was performed, while for the other 36 cases with sufficient tumor cell infiltration, no manual microdissection was carried out. The mutation status of A20 exon 2, 4, 5, 7, and 9 was analyzed by nested-PCR and direct sequencing. Primer sequences and PCR conditions were described previously [3] with a small modification. PCR products were purified by using a PCR purification kit (ZYMO Research, Germany), and 100 ng of purified PCR products were applied for direct sequencing based on capillary electrophoresis (LGC Genomics, Berlin, Germany).

2.5. Statistical analysis

To compare the protein expression of A20 with A20 mutation status, Fisher's exact test was applied. Difference was considered statistically significant when P-value was 0.05 or less. The statistical analysis was performed by using the software package SPSS 19.0 (SPSS, Chicago, USA).

3. Results

3.1. Mutation analysis

The sample set for mutation analysis included 37 lymphomas. CHL had been classified into four histological subgroups: nodular sclerosis (NSCHL, n = 18), mixed cellularity (MCHL, n = 13), lymphocyte-rich (LRHL, n = 1) and lymphocyte-depleted CHL (LDHL, n = 1). Cases without enough tumor tissues for proper evaluation of the background architecture were designated as CHL-not-otherwise-specified (CHL-nos, n = 4). Overall, we detected somatic mutations of A20 in 8 out of 37 (21.6%) cases with Hodgkin Lymphomas (Table 2).

Mutations were mostly concentrated in exon 9 (75%, 6 out of 8 positive cases). Representative mutation profiles in exon 5 and exon 9 are shown in Fig. 1. Interestingly, one patient harbored mutations in exon 2, 5, and 7 simultaneously. One patient only had mutation in exon 5, and one showed mutation in exon 7. In exon 4, however, there was no A20 mutation detectable (Table 2). Most mutations were found in the nodular sclerosis subtype.

3.2. EBV status

Patients with a history of infectious mononucleosis due to Epstein-Barr virus may have an increased risk of HL. In about 40% of classical HL, the HRS cells are latently infected with Epstein-Barr virus (EBV). In such cases, the HRS cells express EBV-encoded RNAs (EBERs) (viral noncoding RNA transcripts). By in situ hybridization